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GLOBAL GENE ANALYSIS OF VARIOUS BIOLOGICAL THREAT AND INFECTIOUS AGENTS USING PBMC: IMPLICATIONS FOR THERAPY AND RAPID DIAGNOSTICS.

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ABSTRACT

Concerns relating to unidentifiable pathogens, that could result from either deliberate or natural mutation processes have prompted studies to find alternative approaches other than the conventional detection methods. Our thesis was that an exposed individual would show gene expression responses unique to the pathogenic agent and prior to onset of the full illness. This study describes our work to establish a library of host responses to pathogenic agents for use to a) identify biological threat agents b) predict the course of impending illness especially for unidentifiable pathogens c) reveal new therapeutic targets. We demonstrate gene expression profiles unique to each pathogenic agent. The gene expression profiles of some agents preceded symptoms of impending illness.

INTRODUCTION

In recent years, there has been a significant change in both the nature and degree of the threat posed by the use of weapons of biological warfare. Substantial gaps in our knowledge concerning mechanisms involved in host immune/defense responses and pathogenesis exists for BW agents such as Bacillus anthracis, Yersinia Pestis, SEB, Cholera, VEE and Brucella species. The threat of terrorist action using biological warfare (BW), chemical or infectious agents has occurred throughout the world. These acts of terrorism are unpredictable and counter efforts have been aimed at rapid, accurate diagnosis and speedy treatment. The approach that we have pursued is to design a system whereby we can determine unique gene expression pattern typical of the impending illness. This approach centers on the fact that peripheral blood lymphoid cells can serve as a reservoir of historical information and can be readily obtained from an exposed individual.

In the past several years, we have been studying host immune responses to biological threat agents that have centered on signal pathways, cell mediators, and evaluation of gene responses upon exposure both in vitro and in vivo to biological threat agents. We have specifically used differential display PCR and gene array technology to determine cellular responses to these agents.

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The approach we have pursued relies on gene expression responses to biological threat agents using PBMC of the exposed individual and we are accumulating a library of these responses to infectious and biological threat agents. Although PBMC may not be the primary target for particular a pathogenic agent, they can respond to a combination of primary and secondary effects and they reflect information, in the form of secreted products as well as gene responses, related to stimuli they have encountered. Gene discovery technology provided the opportunity to examine large numbers of genes simultaneously for the various biological threat agents, both in vitro and in PBMC from animal exposed to the threat agents. The gene array technology offers the potential to determine the degree of individual exposure (and perhaps susceptibility) so that the "worried well" could be separated from the seriously ill.

Based on the developing library of gene responses to biological threat and other pathogenic agents, our ultimate objective is to design gene chips containing relatively few genes (hundreds rather than thousands) that could concisely predict the likely pathogenic agent or modified version of such, the degree of individual exposure and the course of impending illness. This approach aims to provide a tool for defense against biological threats so that resultant panic, morbidity, mortality can be reduced and targets identified for even late-stage treatment modalities.

METHODOLOGY EMPLOYED IN THIS STUDY

Overview. For the past 14 years, our laboratory has been carrying out in vitro and in vivo studies of host responses to staphylococcal enterotoxins as biological threat agents and those studies centered on signal pathways (2-6), cell mediators (5-8), and evaluation of gene expression responses(5). For the latter studies, we have specifically used differential display (DD) -PCR (5) and gene array analysis technology (9)to determine cellular responses to *B. anthracis, B. melitensis, Y. pestis*, staphylococcal enterotoxins (SEs), cholera toxin (CT) and a number of other threat and infectious agents.

Gene discovery technology. Differential display (DD) has been widely used by several groups to identify genes that are altered in response to a particular agonist. There are numerous reports describing the use of global gene analysis to identify critical changes in expression of a few selected genes indicative of specific illnesses. For example, identification of the gene coding for Fetuin was reduced by 45% in liver cirrhosis (10) In some cases specific genes disappeared, such as i) Annexin VI expression in melanoma progression (11) or ii) MIF (macrophage migration inhibitory factor) in metastatic prostate cancer patients (12). These are just a few examples of the utility of DDPCR analysis to identify surrogate markers for a disease state and provide avenues for therapeutic intervention. We have used DDPCR for initial analysis of gene changes with PBMCs.

Gene microarrays. In the studies we describe in this article, we have also utilized gene arrays/microarrays to define gene expression patterns for diagnosis as well as to identify potential new approaches for targeting therapy. Our first approach was to use large commercial screening arrays so that we could design inexpensive custom microarrays to define pathogens in terms of kinetics and dose responses. For in vitro studies, blood was drawn from healthy volunteers and PBMC were obtained by elutriation (13). PBMC were exposed to each biological threat agent (or control) for the designated time period, RNA extracted and purified, RT carried out and the resulting product hybridized onto the gene arrays according to standard procedures (9). The differences in gene expression between control and test for each gene was determined by specialized computer programs. The data were subjected to various software packages for statistical and cluster analysis (for genes within a time period and threat agent)

aimed for these huge data sets (14, 15). Self-organizing maps and other analytical tools were applied to determine patterns of gene expression similarities and differences for each biological threat agent and these analyses also correlated information according to exposure time periods. For in vivo studies, blood was drawn, as described previously (16, 17), from exposed or control monkeys (other of our studies have used piglet models of SE-induced lethal shock) at the designated time periods. In these studies, PBMC were isolated and purified and the same procedure followed as for the in vitro experiments. This approach has provided information in an efficient manner and will facilitate development of a library of genes involved in pathogenesis for each agent examined.

Pathogens. PBMC exposure to each of the pathogens was carried out in the laboratory of the person who is the expert for each biological threat agent. The following is a list of the expert associated with each pathogen. George Ludwig (B. anthracis, VEE), David Hoover (B. melitensis), Luther Lindler (Y. pestis), Neill/Jett (SEs), and Yang (cholera toxin). The cell exposures were carried out using exposure concentrations/doses and other conditions that had been established in their laboratory. For in vitro exposures, the useful time frame ranged from 2-12 hours. In vivo, the time periods reflected the time course of the progression of illness, but blood samples were drawn prior to onset of illness, since gene patterns would be expected to precede the display of illness.

RESULTS AND DISCUSSION

Our laboratories have been carrying out gene expression profiling of the host response to numerous infectious and biological threat agents, however this manuscript will be limited to discussions of studies with anthrax, SEs, LPS, plague, Brucella, anthrax, VEE and cholera toxin. The initial screening used commercial gene arrays studying the host immune response *in vitro* using elutriated human peripheral blood mononuclear cells (PBMC). We have also carried out experiments to compare in vitro and in vivo results by utilizing PBMC obtained from non-human primates challenged with the specific biological threat agent.

Our initial work with global gene analysis studies was directed to determine the extent of the similarities and differences in PBMC responses in vitro to two classical shock-inducing toxins, staphylococcal enterotoxin B vs lipopolysaccharide (LPS), the smallest active unit of endotoxin (5, 9, 18). For these two toxins, the progression of illness is quite similar but we know from the volumes of studies on each toxin that there are some specific differences in production of mediators throughout the course of the illness.

DD-PCR. Our initial studies utilized differential display DD-PCR to examine global changes in host gene responses upon exposure to SEB. At this time, we have examined 100% of mathematically possible gene sequences using combinations of anchored primers (with a poly T-tail) in conjunction with arbitrary primers. One of the most striking aspects of the DD-PCR study is that most of the genes were not altered upon exposure (16 h) to SEB vs control (Figure 1). Anchored primer #3 was used to obtain the data for Figure 1, and the lanes (from the left) are in duplicate pairs for control © vs SEB (16) for each of the 4 sets of arbitrary primers. For the majority of the data, the 4 related bands show equal intensity, indicating that they were not altered in response to toxin exposure. However, there are unmistakable examples of both up- and down-regulated genes in response to SEB in this gel. These clearly altered bands were excised, many have been cloned, purified, sequenced and identified. Approximately 859 genes were found to be >4-fold changed in response to SEB. Of those, most are not yet available from commercial gene banks. This is a much more labor-intensive gene discovery process than is the use of the large screening microarrays, however, a large percentage of the genes it has identified

Figure 1

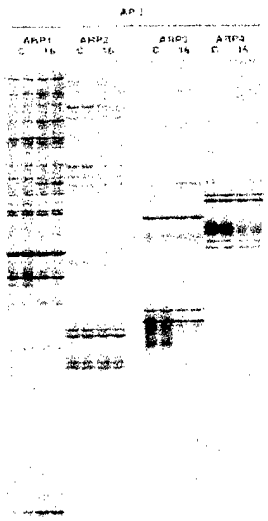

Figure 2

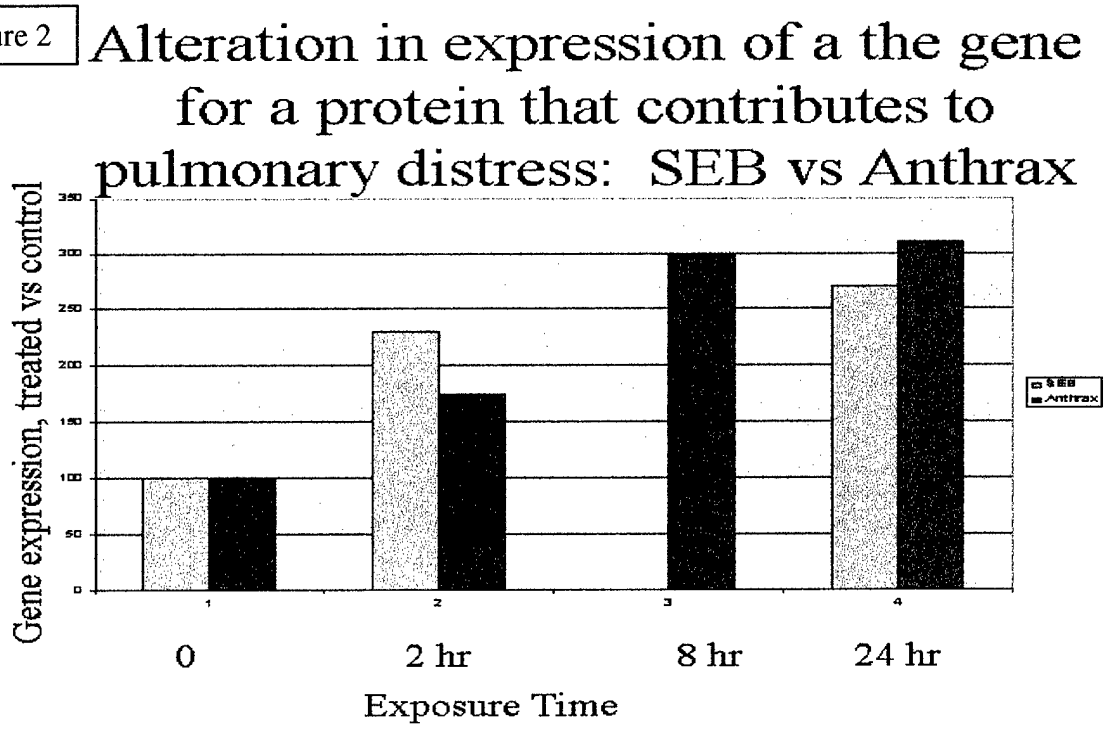

Figure 1. DD-PCR gel electrophoresis study of SEB-induced changes in gene expression using anchored and arbitrary primers as indicated. Each sample had its own control and both control and SEB-treated samples were run in duplicate. As is seen in the figure, the expression levels of most genes did not change. Bands that showed significant alterations in expression were excised, cloned and sequenced for identification through GENBANK.

Figure 2. Changes in Gene Expression in Response to Two Biological Threat Agents. Gene coding for a protein relating to pulmonary distress shows the example of similarities that result

from exposure of PBMC to SEB or LPS in vitro (Note: There was no 8 h sample taken for SEB exposure) Expression of this gene also showed upregulation in monkeys challenged with anthrax. Although for this particular gene, the kinetics of gene expression was similar over the 8-24 h time period, that is frequently not the case. Therefore, study of gene responses in a time-dependent manner is essential, especially for in vivo studies, in order to understand the useful time frame for intervention aimed at potential therapeutic targets.

are genes that are not available commercially at the present time and are not included on screening arrays. In designing our custom arrays (to carry out in-depth analysis of exposure times, doses and potentiating contaminants) we have selected the ~670 genes from screening arrays (altered in response to one or more of the biological threat agents being investigated) and have included the currently cloned and identified 141 genes from DD-PCR. We intend to continue adding these DD-PCR identified genes they become available.

Figure 2 shows an example of SEB or Anthrax-induced changes (relative to controls) in expression of a gene that codes for a protein related to respiratory distress. It is an example of genes showing similar responses to multiple biological threat agents and this expression pattern also was observed in monkeys challenged with anthrax. It is essential to carry out these experiments observing gene alterations at increasing exposure time periods since many genes show time-dependent expression patterns. The kinetic changes in gene expression are especially critical in vivo. An example is that genes coding for cytokines usually display up-regulation at early time periods and the expression levels frequently disappear as time progresses. That is not an unexpected finding, since cytokine production can be seen for brief periods of time following exposure to toxins (5, 9, 16-19).

In contrast to the similarities in gene expression just described, clear differences were seen in response to SEB vs LPS for expression levels of genes coding for numerous cytokines and their accessory molecules, many of the signaling cascade molecules and a variety of other surface adhesion molecules, etc. This probably represents the differences in initial cellular receptors and their linked signal transmission cascades. This study pointed out to us that gene expression changes could show differences in patterns for each agent, but also show similarities relating to common eventual lesions, such as loss of regulation of vascular tone, the hallmark of lethal shock. The unique and common patterns of gene expression were confirmed in SEB-challenged monkeys. Of course, these studies obviously identify new therapeutic intervention sites and, furthermore, predict the time period during which that approach could be usefully targeted.

We have included in vitro exposure of PBMC to B. melitensis as an example of a biological threat agent for our studies aimed at diagnostics. In addition, we have carried out additional studies in which we examined the effects B. melitensis in normal and immunized mouse spleen cells using gene array blots. Genes with altered regulation: stress response genes, oncogenes, and some that encode transcription factors or proteins involved in apoptosis or cell cycling (not previously associated with Brucella-specific immune responses).

PBMC's infected with Venezuelan equine encephalitis virus (VEE) were subjected to gene expression profiling using Gene array blots and Oligonucelotide arrays. In the study two time points namely 1h exposure and 4h exposure were evaluated. It is noteworthy to point out that T cell activation markers were elevated at both the time points. A robust pro inflammatory response was observed with the concomitant up-regulation of both cytokine receptors and its ligands. Many gene of the protein phosphatase family were also up- regulated indicating the indirect role of the VEE in subverting cellular physiology.

This information provided the foundation for the current study of multiple biological threat agents, since identification of course of impending illness (such as loss of regulation of vascular tone, vascular leakage, pulmonary or renal distress, etc) could provide key information should there be exposures to unidentifiable agents. Gene expression responses occur prior to production of their corresponding proteins, and there is frequently a time lag for the concerted action of the causative proteins to result in the demonstration of the lesion. Therefore, gene expression studies offer an early glimpse into the course of the impending illness and shows in a time-dependent manner when a specific therapy regimen might be effective.

Creation of a library of gene expression responses to biological threat agents. We carried out experiments, first in vitro in human PBMC, to determine the pattern of gene expression in response to exposures to B. anthracis, Y. pestis, B. meletensis, SEB and cholera toxin (CT). The latter toxin was chosen essentially to aid in interpretation of the data and for comparisons with the other biological threat agents, since a wealth of information exists about biochemical pathways and their relationship to lesions for CT. For the studies with these 5 pathogens listed above, we accumulated the gene expression response patterns and began to mine from the data the examples of pathogen-specific changes in host immune response gene profiles and identified unique genes that could potentially be used as diagnostic markers and also serve as therapeutic targets. The data amassed in this study are voluminous. We use several clustering analysis programs to group the genes into clusters that are altered significantly induced by each threat agent.

Figure 3 is a condensation of results that shows patterns of gene alterations common to more than one agent and other changes that are unique to a particular agent. In this table, red/pink indicates up-regulated genes while green/blue identifies down-regulated genes. This is but a small set of gene expression changes and they are sorted according to the listed functional activities. As a group, genes coding for cytokines/chemokines were altered by multiple biological threat agents, although specific mediator receptors frequently showed individual responses. The kinetics of gene expression responses is especially important, as pointed out previously, since it reveals potential therapeutic targets that may provide effective intervention at specific time periods during late stages of illness.

NHP-challenge: B. anthracis. We have verified numerous changes in gene expression in non-human primates exposed to B. anthracis at T=0, 24, 48, 72 h post-exposure. We again found, in general, that cytokines appeared primarily at T=24 h while genes involved in apoptosis and cell death were up-regulated at 48-72 h. Many unanticipated changes in gene response have provided potential therapeutic targets for late treatment.

CONCLUSIONS

We conclude that unique gene expression profiles exist for each pathogen that we analyzed. Some gene markers in each pathogen specific gene expression profile correlate very well with current literature. There appears to be a very good correlation between in vitro and in vivo studies using SEB and anthrax. The in vitro gene expression events appear to be time line compressed versions of the in vivo experiments. We were able to relate gene expression patterns to symptoms of impending illness. Through our data mining efforts we have chanced upon many potential therapeutic leads.

Query1

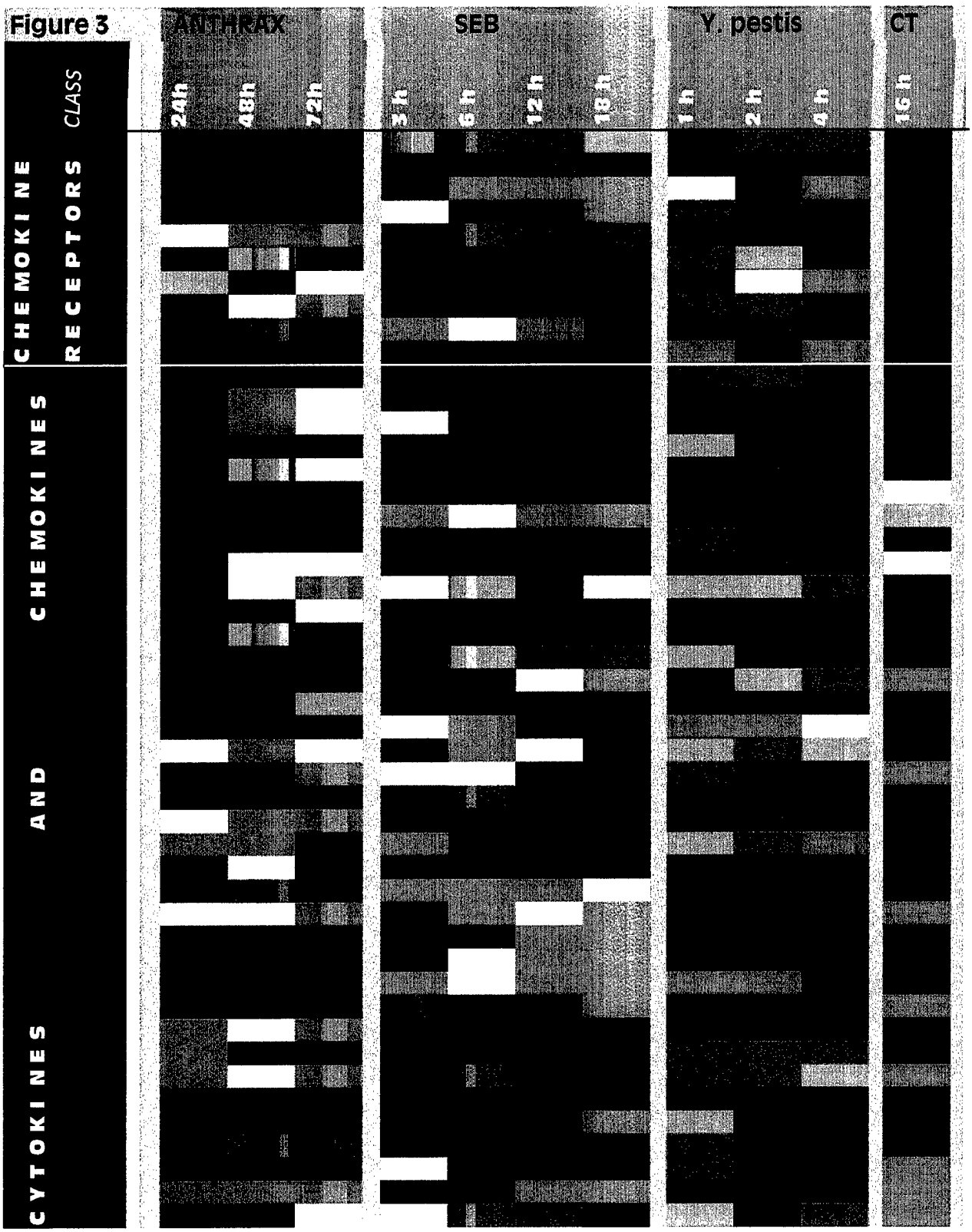

Figure 3. Gene Array Analysis of PBMC Treated with Biological Threat Agen different time periods, usually 2,4,8 hr and for SEB, exposure times were 2,4

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